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Research Article

New insights into the distribution, larval morphology, and genetic diversity of the endangered toad, *Ophryophryne gerti* (Amphibia, Anura, Megophryidae), in Vietnam

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Abstract

Ophryophryne gerti, an endangered toad endemic to the Langbian Plateau, Vietnam, has been known only from a restricted range in Bidoup Nui Ba and Chu Yang Sin National Parks. The conservation and sustainability of the species require knowledge of its true distribution, life history, and genetic diversity. During field surveys from July 2022 to September 2023 on the Di Linh Plateau, the southernmost extent of the Truong Son Range, adult and tadpole specimens of *O. gerti* were collected. A 940 bp fragment of the mitochondrial 12S rRNA–16S rRNA region was sequenced for phylogenetic and population genetic analyses. Our findings extend the species' known range approximately 66 km southward, documenting its presence at lower elevations (500–2000 m). Tadpoles of *O. gerti* are characterized by an oval, depressed body; dorsolateral eyes; sinistral spiracle; pointed tail tip; and keratodont-free oral disc with serrated jaw sheaths. Phylogenetic analyses confirmed that the newly collected Di Linh specimens belong to *O. gerti*, which is closely related to *O. synoria*. Ten haplotypes were detected across populations, with Di Linh showing higher haplotype diversity (Hd = 0.993) but slightly lower nucleotide diversity (π = 0.00465) than Bidoup–Chu Yang Sin (Hd = 0.791; π = 0.00485). Both genetic indices and neutrality tests revealed that populations of this species are threatened in the wild, including Di Linh and well-protected areas such as Bidoup–Nui Ba and Chu Yang Sin National Parks. The Di Linh populations carried excess rare variation or low-frequency polymorphisms due to habitat fragmentation. These results highlight the Di Linh Plateau as an important conservation unit for *O. gerti* and emphasize the need to protect fragmented forest habitats to preserve the species' genetic diversity and adaptive potential.

Key words: 12S rRNA, amphibian conservation, Central Highlands, Di Linh Plateau, endangered species, habitat fragmentation

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Introduction

The Gert's Horned Toad, *Ophryophryne gerti* Ohler, 2003, is currently known only from the Langbian Plateau, Vietnam, at elevations ranging from 700–2000 m (Ohler 2003; Poyarkov et al. 2017). Mahony et al. (2017) proposed a single-genus classification for the subfamily Megophryinae, thereby treating

Ophryophryne as a subgenus of *Megophrys* and referring to the species as *Megophrys* (*Ophryophryne) gerti*. Nevertheless, several subsequent studies (e.g., Chen et al. 2017; Dubois et al. 2021; Lyu et al. 2023) have continued to recognize *Ophryophryne* as a distinct genus. As the present study does not intend to revise the taxonomy of this group, we follow the current scientific name adopted in the Amphibian Species of the World database (Frost 2026) and refer to the species as *Ophryophryne gerti* Ohler, 2003.

Most of the recorded occurrences of the species are within protected areas, specifically Bidoup Nui Ba National Park in Lam Dong Province and Chu Yang Sin National Park in Dak Lak Province. Its habitat is restricted to relatively undisturbed evergreen forests (Poyarkov et al. 2017). Consequently, the species may face significant threats from habitat loss and degradation, which represent the most critical challenges to amphibians in Southeast Asia (IUCN SSC Amphibian Specialist Group 2021). The Langbian Plateau is located in the southern Truong Son Mountain Range of Vietnam, which is one of the regions with a remarkably high rate of threatened amphibian species in the world (Luedtke et al. 2023). Due to its narrow distribution and ongoing decline in habitat quality, *Ophryophryne gerti* is currently classified as endangered on the IUCN Red List (IUCN SSC Amphibian Specialist Group 2021). Determining the species' actual distribution and life history traits is one of the vital first steps toward ensuring its long-term conservation.

Genetic diversity is the foundation of the species' adaptive potential and resilience to environmental change. Studying the population genetics of a threatened species provides valuable insight into its conservation status and can guide the development of effective conservation programs or the establishment of a sustainable management strategy for its genetic resources (Konzen 2014; Willi et al. 2022). Low genetic diversity, often a consequence of small population sizes and habitat fragmentation, can increase susceptibility to inbreeding depression and reduce evolutionary fitness (Paz-Vinas and Blanchet 2015). By identifying distinct genetic units, conservationists can prioritize management actions such as habitat connectivity restoration, translocation, and the design of captive breeding programs. Moreover, genetic data can help detect cryptic population declines and inform reintroduction efforts by ensuring that genetically compatible individuals are selected (Morgan et al. 2008; Schoville et al. 2011). Therefore, integrating population genetics into conservation biology is essential for developing evidence-based and evolutionarily informed approaches to biodiversity preservation. Currently, the study by Poyarkov et al. (2017) provided 12S rRNA–16S rRNA mtDNA sequences of representative individuals for *O. gerti* populations in Bidoup–Nui Ba and Chu Yang Sin. Combining genetic data from other populations across the distribution area of *O. gerti* could provide valuable insights to inform conservation strategies for this endangered species.

To facilitate the conservation of threatened frog species in the southern Truong Son Mountain Range of Vietnam, we surveyed areas south of the Langbian Plateau that lack forest protection and amphibian data. During these surveys, we recorded the presence of *Ophryophryne gerti* outside its previously known range. In this study, we update the species' distributional status, provide the first data on tadpole morphology, and present an initial assessment of the genetic diversity of the newly discovered population compared to that of the known population.

Methods

Sample collection

Specimens (adults and tadpoles) of the species *Ophryophryne gerti* were collected at four forest locations on the Di Linh Plateau, the southernmost point of the Truong Son Range, between July 2022 and September 2023. Site 1 (11.4489°N, 108.0663°E), Gung Re Forest, was surveyed from 22–25 July 2022. Site 2 (11.2764°N, 108.0670°E), Gia Bac Pass, was surveyed from 25–29 August 2022. Site 3 (11.3943°N, 107.7046°E), the foothills of Bao Loc Pass, was surveyed from 6–9 March 2023. Site 4 (11.5513°N,108.1597°E), Brayang Mountain, was surveyed from 26–28 September 2023 (Fig. 2). The surveyed sites were located at elevations ranging from 400 m to 1700 m. Adult samples were collected by hand, and tadpole samples were collected using a fish net. All specimens were photographed alive using a Canon 300D camera. Prior to preservation, tissue samples from the liver (of adults) and tail musculature (of tadpoles) were obtained. The tissue samples were stored in 99% ethanol. All voucher specimens were then preserved with 10% formalin.

Morphological examination and measurement

Measurements of adult specimens were made with a dial caliper to the nearest 0.1 mm. Morphometrics of adult frogs and character terminology follow Mahony (2011) and Mahony et al. (2013), including: **SVL** snout to vent length; **HW** head width at commisure of jaws; **HL** head length from tip of snout to rear of jaws; **ED** diameter of exposed portion of eyeball; **TYD** horizontal diameter of tympanum; **TYE** distance from anterior edge of tympanum to posterior corner of eye; **SL** snout length from tip of snout to anterior corner of eye; **EN** distance from front of eye to center of nostril; **IUE** interupper eyelid width, i.e., shortest distance between upper eyelids; **IN** internarial distance; **UEW** maximum upper eyelid width; **FAL** forearm length measured from elbow to wrist; **HAL** hand length measured from wrist to tip of third digit; **TL** thigh length from cloaca to knee; **FOL** foot length, measured from proximal end of inner metatarsal tubercle to tip of fourth digit. Sex was determined by direct observation of calling in life.

The staging of the tadpoles was in accordance with Gosner (1960). Dial calipers were used to measure four tadpole specimens to the nearest 0.1 mm. Morphological measurements followed Altig and McDiarmid (1999) and Pezzuti et al. (2021), including: **TL** total length; **BL** body length; **TaL** tail length; **MTH** maximum tail height; **IOD** interorbital distance; **IND** internostril distance; **TMH** tail muscle height; **TMW** tail muscle width; **BW** body width; **BH** body height; **ESD** eye–snout distance; **END** eye–nostril distance; **NSD** nostril–snout distance; **ED** eye diameter; **ND** nostril diameter; **SSD** snout–spiracular distance; **ODW** oral disc width; **DFH** dorsal fin height; **VFH** ventral fin height; **SL** spiracle length; **RSU** transverse rows of submarginal papillae on upper labium; **RSL** transverse rows of submarginal papillae on lower labium (Gosner 1960; McDiarmid and Altig 1999; Pezzuti et al. 2021). The tooth formulas (**LTRF**) were determined according to Altig and McDiarmid's methodology (1999).

Molecular study

Genomic DNA was isolated from tissue samples using the Omega BIO-TEK tissue kit following the manufacturer's protocol. Approximately 940 base pairs of the mitochondrial 12S rRNA–16S rRNA gene fragment were amplified using the primer pair LX12SN1 (5'-TACACACCGCCCGTCA-3') and LX16S1R (5'-GACCTGGATTACTCCGGTCTGAACTC-3') (Zhang et al. 2008). Each 40 µL PCR reaction contained 20 µL of MyTaq PCR Master Mix (Bioline), 16 µL of ultrapure water, 0.8 µL of BSA (Euromedex), 0.6 µL of each primer (3 µM), and 2 µL of DNA template. PCR amplifications were performed in a PTC-100 thermal cycler (Bio-Rad) under the following conditions: an initial denaturation step of 2 min at 92 °C, followed by 35 cycles of 45 s at 92 °C, 45 s at 52 °C, and 1 min at 72 °C, with a final extension of 5 min at 72 °C and a hold at 4 °C. PCR products were visualized on 1–2% agarose gels, and the most intense amplicons were selected for bidirectional sequencing by 1ST BASE (https://base-asia.com/). Electropherograms were visually inspected and edited using Chromas v.2.6.6, and sequences were aligned using MUSCLE as implemented in MEGA 7 (Kumar et al. 2016). Genetic divergence among sequences was estimated in MEGA 7 using the *p*-distance model (Kumar et al. 2016). All newly generated sequences were deposited in GenBank under accession numbers PX924301 to PX924322 (n = 22 sequences). To infer the phylogenetic position of the newly discovered population, additional 12S rRNA–16S rRNA sequences were retrieved from GenBank for 28 species belonging to the genera *Ophryophryne* Boulenger, 1903, *Xenophrys* Günther, 1864, and *Brachytarsophrys* Tian & Hu, 1983, with *Megophrys nasuta* Schlegel, 1858 used as the outgroup (Poyarkov et al. 2017). Phylogenetic reconstructions were conducted under the Maximum Likelihood (ML) framework using IQ-TREE via the IQ-TREE web server (Nguyen et al. 2015; Trifinopoulos et al. 2016). The best-fit substitution model was selected using ModelFinder (Chernomor et al. 2016; Kalyaanamoorthy et al. 2017) based on the Bayesian Information Criterion (BIC). The TIM2+F+G4 model was identified as the optimal model for ML analyses (BIC = 11646.584, −lnL = 5486.254). Node support was assessed using 1000 ultrafast bootstrap replicates (Minh et al. 2013). Resulting trees were visualized and edited using FigTree v.1.4.3 (https://tree.bio.ed.ac.uk/software/figtree/). To provide an initial assessment of genetic diversity and population structure of *Ophryophryne gerti*, population genetic analyses were conducted based on the 12S rRNA–16S rRNA sequence dataset. The number of haplotypes (h), haplotype diversity (Hd), and nucleotide diversity (π) were calculated using DnaSP v.6 (Rozas et al. 2017). The F_{st} value was calculated from the AMOVA results to measure population differentiation. Genetic relationships among haplotypes and their geographic distribution, with particular emphasis on tadpole samples, were visualized by constructing a haplotype network in PopArt v.1.7 (Leigh and Bryant 2015) using input files generated in MEGA and the TCS network algorithm (Clement et al. 2002). To investigate historical demographic processes and deviations from neutral evolution, neutrality tests including Tajima's *D* (Tajima 1989), Fu and Li's *D**, and Fu and Li's *F** (Fu and Li 1993) were performed in DnaSP. These statistics were used to infer potential signals of population expansion, contraction, or selective pressures acting on the mitochondrial DNA sequences of the studied populations.

Distribution map

A species distribution map (Fig. 2) was created in QGIS using the ESRI World Topographic basemap layer. The known distribution data of *Ophryophryne gerti* were downloaded from the IUCN Red List (IUCN SSC Amphibian Specialist Group 2021).

Results

Species confirmation

Sequences generated from the tadpole and adult specimens were identical to those from sequences of *Ophryophryne gerti* (99% ± 0.01) reported by Poyarkov et al. (2017) (GenBank accession numbers KY425365–KY425378). In addition, morphological characteristics of adult specimens (*n* = 3, males) match the descriptions of Ohler (2003) and Poyarkov et al. (2017) (Table 1, Fig. 1E). SVL: 33.4–41.8 mm; head wider (HW: 10.1–13.1 mm) than long (HL: 9.6–12.9 mm); eyes large (ED/HL: 32–28%); horizontal eye diameter less than twice as long as the maximum tympanum diameter (TYD/ED: 69.7–73.1%); forelimbs slender, forearm moderately long (FAL/SVL: 19.5–26.6%); hindlimbs slender, shank length less than half of the snout to vent length (SHL/SVL: 46.7–51.2%); thighs shorter than shanks (SHL/TL: 103.1–105.3%) and feet (FOL/TL: 134.8–147.9%); grey or black-brown nuptial pads on males in life; strong dorsolateral glandular ridge from above each shoulder to approximately 4/5 of the distance between the axilla and groin, connecting with posterior tips of the " >–< "-shaped parietoscapular-sacral glandular ridge; skin on the dorsum and sides of the body granular, with numerous small and medium-sized tubercles and larger warts.

Table 1. Measurements (mm) of *Ophryopryne gerti* adults from the Di Linh Plateau (DL, this study) and Langbian Plateau (Poyarkov et al. 2017).

Specimen ID	DL17A	DL48A	DL62A	Poyarkov et al. 2017
Sex	male	male	male	males
SVL	33.39	41.83	38.8	31.7–42.2
HL	10.06	13.1	12.58	9.0–12.0
HW	9.65	12.9	11.78	9.2–12.0
ED	3.72	4.09	3.86	3.8–5.3
TYD	2.65	2.85	2.82	1.8–3.1
TYE	1.31	2.68	1.84	1.7–2.5
SL	2.45	4.11	3.18	2.6–3.8
EN	1.17	1.64	1.38	0.7–1.8
IUE	3.62	3.83	3.29	2.2–4.1
IN	2.34	3.11	2.87	1.9–3.4
UEW	2.78	3.44	3.12	2.2–3.8
FAL	8.89	8.15	8.26	7.2–11.0
HAL	8.60	11.51	10.18	7.2–10.1
SHL	17.99	19.52	19.49	14.4–24.6
TL	17.09	18.82	18.9	13.9–23.3
FOL	14.50	16.98	15.74	11.2–18.8

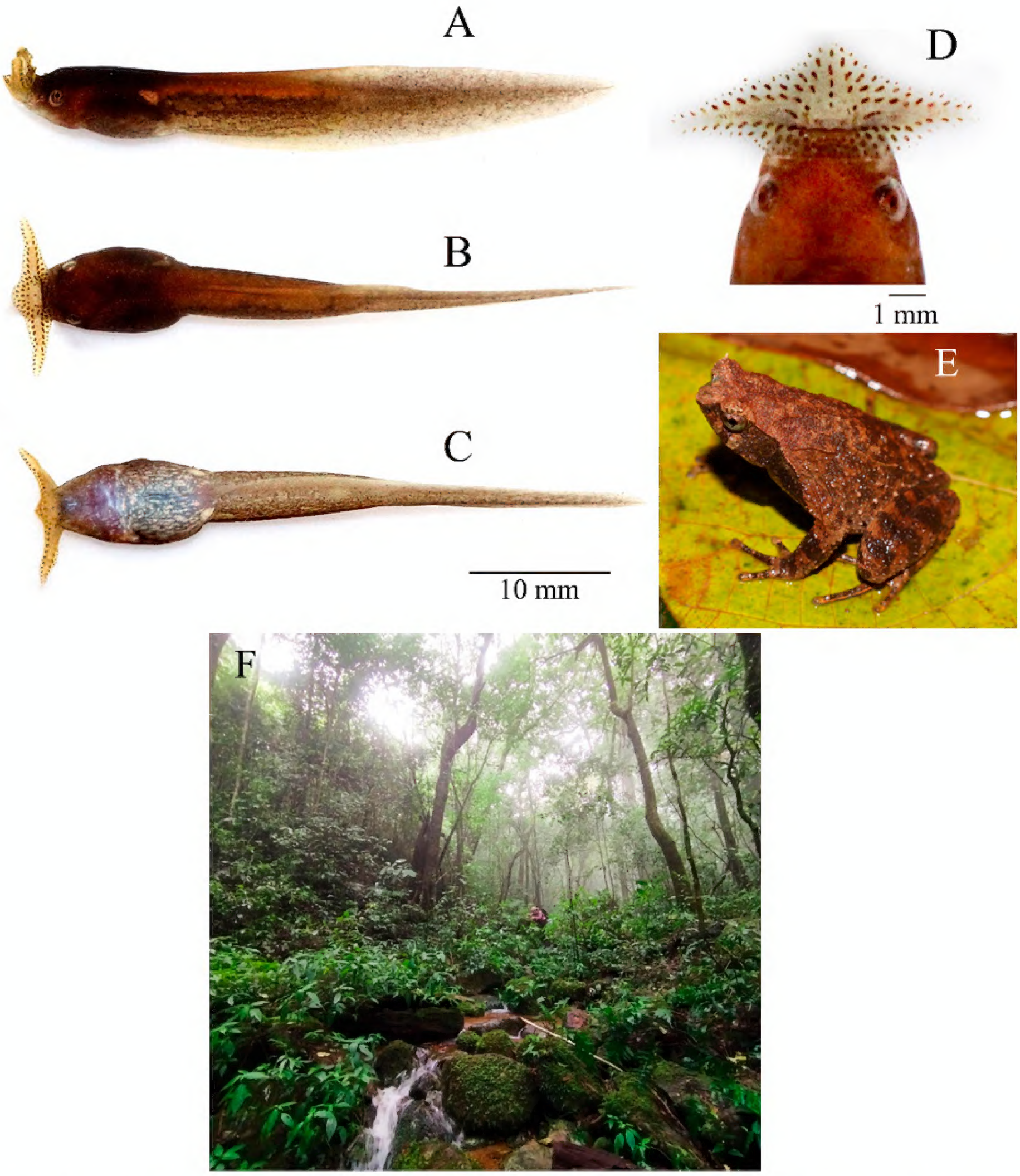

Figure 1. A–D. External morphology of the *Ophryophryne gerti* tadpole (stage 36) in life: **A.** Lateral view; **B.** dorsal view; **C.** Ventral view; **D.** Oral disc. **E.** Adult *O. gerti*; **F.** Habitat of *O. gerti* in the Di Linh Plateau, Lam Dong Province, Vietnam.

Ophryophryne gerti tadpole description

The following description of *Ophryophryne gerti* tadpoles is based on seven specimens at stage 25 (*n* = 5), 26 (*n* = 1), and 36 (*n* = 1) (Fig. 1, Table 2).

In dorsal view (Fig. 1A): body oval-shaped and depressed (BH/BW = 0.83 ± 0.04 at stage 25–26; and = 0.79 at stage 36); smoothly tapering toward base of oral funnel in both lateral and dorsal views; eyes round, medium-sized (ED/BWE = 0.26 ± 0.01 at stage 25–26; and = 0.25 at stage 36), located dorsolaterally (IOD/BWE = 0.86 ± 0.03

Table 2. Measurements (mm) of *Ophryopryne gerti* tadpoles from the Di Linh Plateau, Lam Dong Province, Vietnam.

Stage	25 (*n* = 5)	26 (*n* = 1)	36 (*n* = 1)
TL	41.11 ± 4.70	42.65	42.49
BL	10.23 ± 0.92	11.06	11.16
TaL	30.88 ± 3.86	31.59	31.33
MTH	6.61 ± 0.75	7.36	6.26
IND	3.37 ± 0.24	3.45	3.48
IOD	3.95 ± 0.34	4.27	4.40
TMH	4.13 ± 0.55	4.61	4.30
TMW	3.64 ± 0.73	3.75	3.94
BW	6.14 ± 0.69	6.67	7.11
BWN	3.90 ± 0.22	3.86	4.29
BWE	4.58 ± 0.28	4.84	5.36
BH	5.04 ± 0.59	5.72	5.60
ESD	2.80 ± 0.20	2.89	2.99
END	0.98 ± 0.11	1.10	1.17
NSD	1.74 ± 0.24	1.69	1.81
ED	1.17 ± 0.08	1.28	1.35
ND	0.27 ± 0.06	0.32	0.31
SSD	5.66 ± 0.45	5.88	6.23
ODW	8.81 ± 1.42	9.13	6.94
DFH	1.60 ± 0.19	1.90	1.67
VFH	1.48 ± 0.13	1.64	1.53
RSU	4	4	4
RSL	5–6	5	5

at stage 25–26; and = 0.82 at stage 36), and directed dorsolaterally; nostrils tubular with serrated rims, opening round, medium-sized (ND/BL = 0.03 ± 0.003 at stage 25–26; and = 0.03 at stage 36), located laterally, directed laterally and positioned closer to eye than snout (NSD/ESD = 0.60 ± 0.03 at stage 25–26; and = 0.61 at stage 36); neuromasts are visible both on body and tail.

In lateral view (Fig. 1B): body globular depressed (BH/TL = 0.13 ± 0.01 at stage 25–26; and = 0.13 at stage 36); spiracle sinistral, positioned laterally, directed posterodorsally, opening at middle third of body (SSD/BL = 0.55 ± 0.02 at stage 25–26; and = 0.56 at stage 36), inner wall fused to body, with distal portion free from body; tail intermediate height (MTH/TAL = 0.22 ± 0.02 at stage 25–26; and = 0.20 at stage 36); musculature robust (TMH/BH = 0.81 ± 0.04 at stage 25–26; and = 0.77 at stage 36) with tail tip pointed; dorsal fin of low height (DFH/TAL = 0.05 ± 0.01 at stage 25–26; and = 0.05 at stage 36), originating posterior to body–tail junction, with convex external margin at middle of trunk, then gradually descending to tail tip; ventral fin of low height (VFH/TAL = 0.05 ± 0.004 at stage 25–26; and = 0.05 at stage 36), originating posterior to body–tail junction, with a slightly convex external margin at middle of trunk, then tapering tip; dorsal fin higher than ventral (DFH/VFH = 1.14 ± 0.04 at stage 25–26; and = 1.09 at stage 36).

In ventral view (Fig. 1C): gut tube invisible due to the robustness of rectus abdominis muscle; vent tube opening medially, positioned anterior to ventral fin and unattached to the ventral fin.

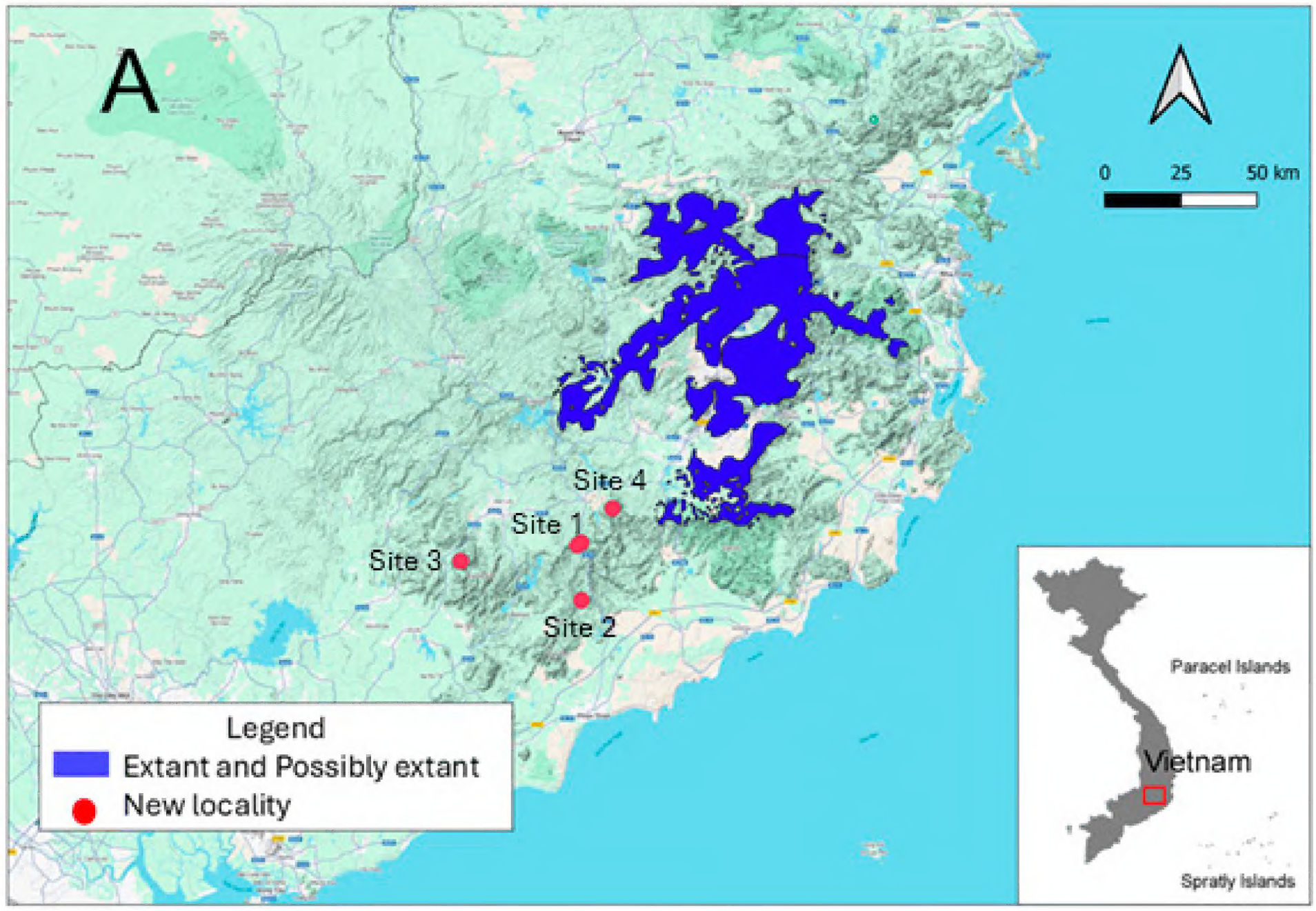

Figure 2. Distribution of *Ophryophryne gerti* and habitat in the Di Linh Plateau. **A.** The updated distribution range of *Ophryophryne gerti*; **B.** Agriculture activities in the Di Linh Plateau. The solid blue area represents the presumed range and areas where this species may possibly be extant according to IUCN Red List data.

Oral disc (Fig. 1D) wide (ODW/BW = 1.42 ± 0.12 at stage 25–26; and = 0.98 at stage 36), terminal, shaped funnel-liked, opening anteriordorsally, the lower lip fully expanded to bi-triangular shaped in live; four and five to six transverse rows of submarginal papillae present on upper and lower labium, respectively, and

the submarginal papillae smaller in outermost rows on upper and lower lips; a row of five to eight transparent submarginal papillae present as "U"-shaped with a concave side directed to lower jaw; keratodonts absent; the upper jaw sheath serrated and with medial notch, whereas the lower jaw sheath serrated, lacking a medial notch, and forming arch shape.

Color in life

On dorsal side, tadpoles were covered brownish-orange pattern that extended to anterior part of tail with irregular dark brown speckles, and a dark brown patch present from nare to nare (or present as a dark brown bar with median gap); neuromasts distinctly visible. On lateral side, the brownish-orange pattern extends to above horizontal level of the spiracle; tail musculature dark brown; tail fins semi-translucent; white and dark brown speckles occur on tail. On ventral side, belly semi-translucent with dense milky white spots, the red gill still visible through the ventral skin. Oral disc light brown with dark brown submarginal papillae.

Color in preservative

The color of tadpoles in preservative is paler and absent milky white spots when compared to color in life. The body is brown uniform or with darker speckles on dorsum. Belly white and peppering brown spots. Tail fins translucent to brown with dark blotches. Tail musculature light brown with darker patches.

Comparisons

The tadpoles of *O. gerti* differ from those of other *Ophryophryne* species previously described by having four transverse rows of submarginal papillae on upper lip (vs. two to three in *O. elfina* and two in *O. microstoma*); five to six transverse rows of submarginal papillae on lower lip (vs. four in *O. elfina* and *O. microstoma*); tail tip pointed (vs. bluntly rounded in *O. elfina*); and neuromasts visible in preservative (vs. invisible in *O. elfina* and *O. microstoma*) (Grosjean 2003; Poyarkov et al. 2017).

Ecology

Both adult individuals and tadpoles have been recorded inhabiting small forest streams characterized by rocky and gravelly substrates. These streams are situated within evergreen broadleaf and mixed broadleaf–bamboo forests (Fig. 1F). At disturbed stream sites lacking forest cover on either bank, the species was absent during our surveys. Tadpoles were documented in both the dry season (March) and the rainy season (July–September), suggesting that the species has the capacity to reproduce year-round. Larvae were typically concentrated in shallow waters with abundant leaf litter.

Distribution of *Ophryophryne gerti*

O. gerti is currently known from the Langbian Plateau, southern Annamites. Our new record sites are approximately 66.0 km from the species' known range and at a lower elevation than previously reported, according to the

IUCN Red List data, expanding the known extent of occurrence for the species far south (Fig. 2A). The species is now known to occur between 500 and 2000 m asl.

Phylogeny and genetic diversity of *Ophryophryne gerti*

The maximum likelihood (ML) phylogenetic analysis based on the mitochondrial 12S rRNA–16S rRNA fragment yielded a well-resolved topology, with most nodes receiving bootstrap support values exceeding 70% (Fig. 3A). All newly collected specimens (*n* = 22) from the Di Linh Plateau clustered unequivocally with previously published sequences of *Ophryophryne gerti* from the Langbian Plateau (*n* = 14, Poyarkov et al. 2017), with strong bootstrap support (97%), confirming their conspecific status. Within the genus *Ophryophryne*, *O. gerti* was recovered as the sister taxon to *O. synoria*, with maximal bootstrap support (100%) (Fig. 3A). Pairwise genetic distance analyses further supported this relationship, showing low divergence between *O. gerti* and *O. synoria* (*p*-distance = 0.05 ± 0.01) based on the 12S rRNA–16S rRNA dataset. The genetic distances of all species on the phylogenetic tree are presented in Table 3. The ML tree also revealed a strongly supported clade (BP = 100%) comprising *O. gerti*, *O. synoria*, *O. elfina*, *O. hansi*, *O. microstoma*, and *O.* cf. *poilani*, corresponding to the genus *Ophryophryne*. Within this clade, *O. gerti*, together with *O. synoria* and *O. elfina*, formed a geographically coherent subclade distributed in the Langbian Plateau of southern Vietnam and adjacent regions of Cambodia (Poyarkov et al. 2017). This southern Indochinese clade was more distantly related to a second subclade consisting of *O. hansi*, *O. microstoma*, and *O.* cf. *poilani*, which are distributed in southern China and northern to central Indochina (Poyarkov et al. 2017), with a mean genetic divergence of 0.11 ± 0.01.

From the sequences analyzed, *Ophryophryne gerti* has a total of 10 haplotypes, including a high-frequency central haplotype (H5) that connects to surrounding low-frequency satellite haplotypes by one to two mutations (Table 4, Fig. 3B). The central haplotype H5 is shared in the Di Linh and Bidoup–Nui Ba areas. Of the low-frequency haplotypes around H5, there is one shared H9 haplotype in the two areas of Bidoup–Nui Ba and Chu Yang Sin; all of the remaining low-frequency haplotypes occur in only one location. This result indicated that the *O. gerti* populations in Di Linh and Bidoup–Nui Ba are closely related through a high frequency of haplotype H5. The genetic differences between the two populations in the Di Linh and Bidoup–Chu Yang Sin plateaus were low, with F_{st} = 0.09. However, the population of this species in the Di Linh Plateau (including the Bao Loc area) showed a distinct genetic diversity by containing five unique haplotypes.

Genetic diversity indices of *Ophryophryne gerti* based on mitochondrial regions 12S rRNA–16S rRNA by region are shown in Table 4. The nucleotide diversity index (π) of *O. gerti* in Di Linh was lower than that in Bidoup–Chu Yang Sin, but the haplotype diversity index (Hd) in Di Linh was higher than that in Bidoup–Chu Yang Sin. This showed that there were more different alleles in the Di Linh population than in Bidoup–Chu Yang Sin, but the nucleotide composition between these alleles in Di Linh was not as divergent as that of the alleles appearing in Bidoup–Chu Yang Sin. The Fu and Li's *D**,

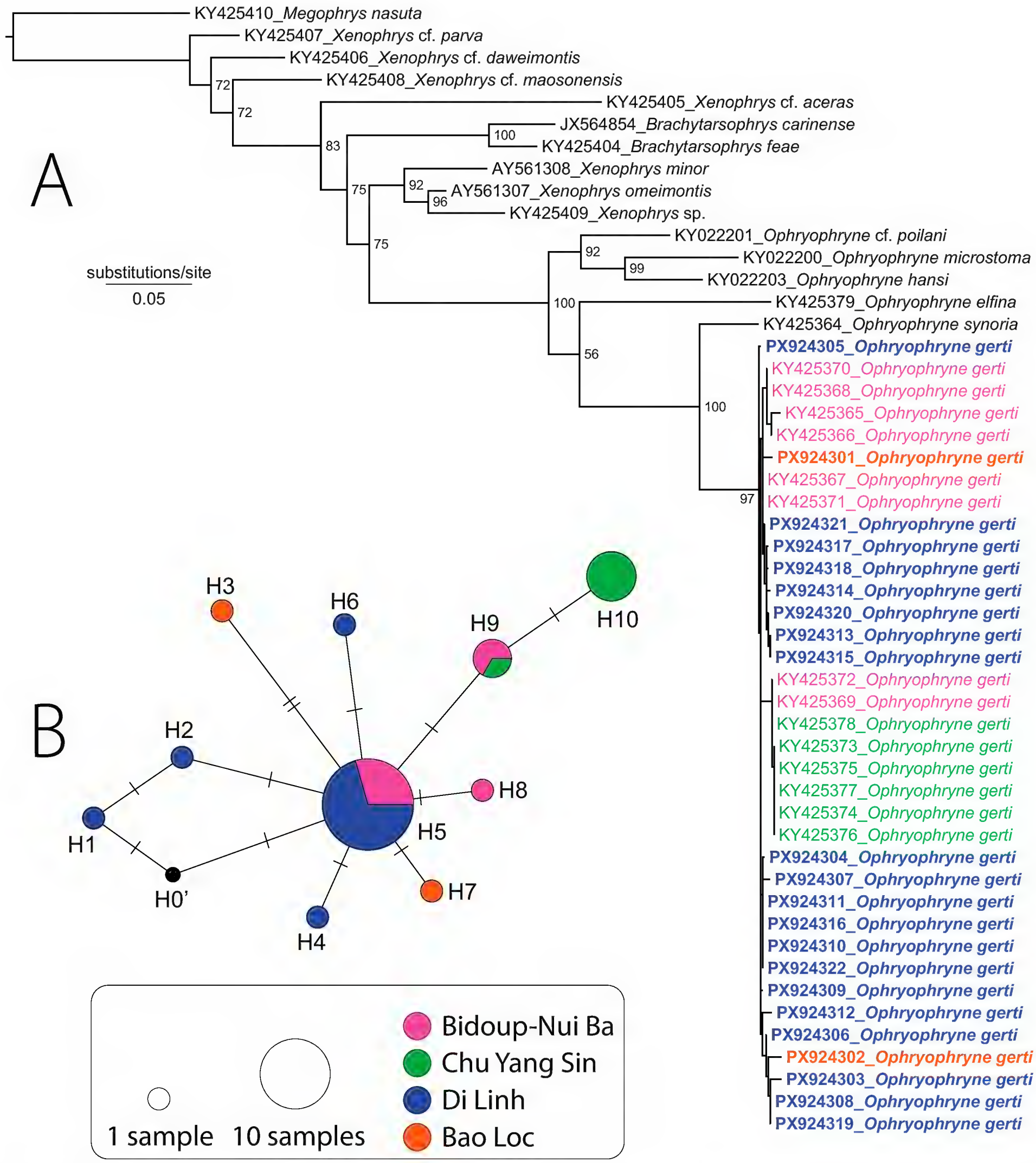

Figure 3. Genetic analyses based on 12S rRNA–16S rRNA mtDNA presenting at **A.** ML phylogenetic tree of *Ophryophryne* spp. with bootstrap supports at nodes (newly collected specimens from Di Linh and Bao Loc are in bold text); **B.** The haplotype network of *Ophryophryne gerti* showing various colors among areas.

Fu and Li's *F**, and Tajima's *D* tests of the samples in Bidoup–Nui Ba and Chu Yang Sin exhibited positive values, while negative values were observed in Di Linh. It suggested an excess of common variation in a region, which can be consistent with balancing selection or population contraction in Bidoup–Nui Ba and Chu Yang Sin; these trends were significantly different ($P < 0.05$). In contrast, there was an excess of rare variation or low-frequency polymorphisms in the Di Linh Plateau.

Table 3. Estimates of divergence over sequence pairs between species in the phylogenetic tree. Standard error estimates are shown above the diagonal with 1,000 bootstraps.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
1. *Megophrys nasuta*		0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02
2. *Xenophrys* cf. *parva*	0.13		0.01	0.01	0.02	0.02	0.02	0.01	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02
3. *Xenophrys* cf. *daweimontis*	0.14	0.05		0.01	0.02	0.02	0.02	0.01	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02
4. *Xenophrys* cf. *maosonensis*	0.15	0.09	0.07		0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02
5. *Xenophrys* cf. *aceras*	0.16	0.14	0.13	0.13		0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02
6. *Brachytarsophrys carinense*	0.14	0.10	0.11	0.12	0.13		0.01	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02
7. *Brachytarsophrys feae*	0.17	0.11	0.11	0.12	0.14	0.05		0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02
8. *Xenophrys minor*	0.16	0.09	0.09	0.11	0.13	0.10	0.09		0.01	0.01	0.02	0.02	0.02	0.02	0.02	0.02
9. *Xenophrys omeimontis*	0.15	0.09	0.09	0.10	0.13	0.09	0.08	0.05		0.01	0.02	0.02	0.01	0.02	0.02	0.02
10. *Xenophrys* sp.	0.16	0.10	0.10	0.11	0.14	0.09	0.10	0.06	0.04		0.02	0.02	0.02	0.02	0.02	0.02
11. *Ophryophryne* cf. *poilani*	0.17	0.11	0.12	0.11	0.15	0.13	0.13	0.10	0.10	0.10		0.01	0.01	0.01	0.02	0.02
12. *Ophryophryne microstoma*	0.16	0.10	0.12	0.10	0.17	0.12	0.12	0.09	0.10	0.11	0.07		0.01	0.02	0.02	0.02
13. *Ophryophryne hansi*	0.18	0.11	0.13	0.12	0.18	0.12	0.12	0.10	0.09	0.10	0.08	0.06		0.01	0.02	0.02
14. *Ophryophryne elfina*	0.16	0.11	0.14	0.16	0.19	0.12	0.13	0.11	0.11	0.12	0.08	0.10	0.09		0.02	0.02
15. *Ophryophryne synoria*	0.19	0.14	0.14	0.15	0.18	0.13	0.14	0.14	0.14	0.14	0.10	0.11	0.13	0.10		0.01
16. *Ophryophryne gerti*	0.16	0.14	0.14	0.15	0.18	0.13	0.14	0.14	0.14	0.14	0.10	0.11	0.12	0.09	0.06	

Table 4. Index of genetic diversity of *Ophryophryne gerti* based on the mitochondrial gene region 12S rRNA–16S rRNA.

Genetic diversity parameters and indicators	Di Linh Plateau	Bidoup–Chu Yang Sin
Number of sequences	22	14
Number of haplotypes	7	4
Nucleotide Diversity (π)	0.00465	0.00485
Diverse haplotype (Hd)	0.99	0.79
Fu and Li's *D** test	−2.62 *P < 0.05*	0.02 *P < 0.05*
Fu and Li's *F** test	-2.85 *P < 0.05*	0.14 *P < 0.05*
Tajima's *D* test	−2.06 *P < 0.05*	0.40 *P < 0.05*

Discussion

The discovery of *Ophryophryne gerti* in the Di Linh Plateau significantly extends the known distribution range of this endangered species, providing essential data for conservation planning. Occurrences at lower elevations and outside established protected areas highlight the importance of integrating fragmented forest habitats into regional conservation strategies. The presence of *O. gerti* in Di Linh also suggests a distribution pattern similar to that of its sister species *O. synoria*, with both species occurring on the Langbian Plateau and the lower areas of Di Linh (Poyarkov et al. 2017). Our observations of *O. gerti* from lower elevations considerably expand the known altitudinal range of the species and bring it into closer ecological proximity with *O. synoria*. In this study, we recorded both species in a stream at the foothill area of Bao Loc Pass, suggesting that their distributions may locally overlap and even occur syntopically. This indicates a broader overlap in both elevational range and habitat conditions than previously recognized. Such findings further strengthen the evidence for a close relationship between Langbian and Di Linh, which are two geographically

adjacent plateaus formed on similar geological grounds (Stattersfield et al. 1998; Rundel 2000; Bain and Hurley 2011).

The tadpole morphology of *Ophryophryne gerti* corresponds to a lotic-neustonic ecomorphotype, which is typically adapted to flowing-water environments (Altig and Johnston 1989). Such morphological specialization is commonly associated with headwater stream habitats, where current velocity, substrate structure, and organic matter retention jointly shape larval microhabitats. Previous records indicate that *O. gerti* occurs predominantly in well-preserved evergreen forests (Poyarkov et al. 2017; IUCN SSC Amphibian Specialist Group 2021). Consistent with these observations, the present study recorded the species in forested stream sections, where tadpoles were found sheltering within accumulations of leaf litter on the streambed. Leaf litter appears to play an important role as a microhabitat component for the larval stage of *O. gerti*, providing refuge from hydraulic disturbance as well as potential food resources. Similar patterns have been reported in other tropical forest systems, where leaf litter significantly influences tadpole assemblage structure and abundance, including studies on *Bokermannohyla hylax* and *Aplastodiscus leucopygius* in the Atlantic Forest (Bertoluci et al. 2021). These findings suggest that litter-rich microhabitats may be a key ecological requirement for stream-breeding amphibians with specialized larval traits.

In the Di Linh Plateau, however, no formal protected areas have been established, and agricultural activities—particularly coffee cultivation—are expanding rapidly. Field observations indicate that stream reaches bordered by coffee plantations exhibit higher flow intensity and reduced amounts of organic debris compared to streams within natural forests. Such changes are consistent with documented effects of riparian forest loss, including reduced input of leaf litter and coarse organic matter, increased current velocity, and enhanced substrate erosion in headwater streams. These structural alterations may negatively affect amphibians whose larval stages depend on stable, low-energy microhabitats within forested streams (Muto et al. 2009; Souza et al. 2013; Bleich et al. 2014). Although this study did not quantitatively assess differences in the distribution or abundance of *O. gerti* between disturbed and undisturbed stream sections, the observed association between tadpoles and intact riparian forest, together with established links between riparian degradation and stream structural change, suggests that habitat modification may pose a risk to the species. Given its apparent ecological specialization, *O. gerti* may be particularly sensitive to changes in riparian vegetation and stream physical structure.

The phylogenetic analyses based on the mitochondrial 12S rRNA–16S rRNA fragment clearly confirmed that all newly collected specimens from the Di Linh Plateau belong to *Ophryophryne gerti* and are conspecific with populations previously reported from the Langbian Plateau. The high bootstrap support for the monophyly of *O. gerti* (97%) and its sister relationship with *O. synoria* (100%) is consistent with earlier phylogenetic studies of the genus *Ophryophryne* (Poyarkov et al. 2017). Together with *O. elfina*, these taxa form a well-supported clade endemic to the Langbian Plateau and adjacent areas of southern Vietnam and Cambodia, highlighting the importance of this montane region as a center of endemism for megophryid frogs. The relatively low genetic divergence between *O. gerti* and *O. synoria* (*p*-distance ~ 0.05) compared to the deeper divergence separating southern Indochinese taxa from lineages distributed in southern China and northern Indochina (~ 0.11) suggests a more recent diversification

of Langbian Plateau lineages, possibly linked to regional geological or climatic processes. Such shallow divergences have been reported for other amphibian taxa restricted to montane systems in southern Vietnam (Poyarkov et al. 2017), emphasizing the role of highland refugia in shaping amphibian diversity.

Population genetic analyses revealed a relatively simple haplotype structure for *O. gerti*, characterized by a single high-frequency central haplotype (H5) shared between Di Linh and Bidoup–Nui Ba populations, surrounded by multiple low-frequency, mostly locality-specific haplotypes. This star-like haplotype network pattern, together with the low pairwise F_{st} value (0.09), indicates recent or ongoing genetic connectivity between populations or a shared demographic history. At the same time, the presence of several unique haplotypes in the Di Linh Plateau suggests some degree of genetic differentiation and local diversification. Genetic diversity indices further revealed contrasting patterns between regions. Although nucleotide diversity (π) was lower in the Di Linh population compared to Bidoup–Chu Yang Sin, haplotype diversity (Hd) was higher in Di Linh. This combination of high haplotype diversity but low nucleotide divergence is commonly interpreted as an accumulation of multiple closely related haplotypes, potentially arising from recent population expansion, localized mutation accumulation, or demographic instability. Similar patterns have been reported in other amphibian species experiencing habitat fragmentation or environmental stress (Chen et al. 2013; Keely et al. 2015; Langone et al. 2016).

Neutrality tests provide additional insights into the demographic history of *O. gerti*. The positive values of Tajima's *D*, Fu and Li's *D**, and Fu and Li's *F** observed in Bidoup–Nui Ba and Chu Yang Sin populations may reflect an excess of intermediate-frequency variants, which can be consistent with demographic contraction or population structure within relatively stable forest habitats. In contrast, the negative Tajima's *D* values detected in the Di Linh population indicate an excess of rare haplotypes, suggesting a departure from mutation–drift equilibrium. Such a pattern may arise under several non-mutually exclusive scenarios, including recent demographic expansion following a bottleneck, founder effects, or selective processes acting on mitochondrial lineages (Templeton 1980; Provine 2004; William 2009). Importantly, the Di Linh Plateau is known to experience substantial anthropogenic pressure, including habitat loss, fragmentation, and landscape modification due to agricultural expansion and infrastructure development (People's Committee of Lam Dong Province 2019). The genetic signature observed in the Di Linh population—namely, the presence of multiple low-frequency haplotypes derived from a common central haplotype—may therefore reflect recent demographic disturbances rather than long-term evolutionary processes. Under such conditions, rare haplotypes can accumulate rapidly against a background of limited overall sequence divergence, as expected following founder or bottleneck events. Taken together, our results suggest that while *O. gerti* populations across the Langbian and Di Linh plateaus remain genetically closely related, the Di Linh population shows signals of localized genetic differentiation and demographic instability. These findings underscore the conservation importance of remnant forest habitats in the Di Linh Plateau, which may function as reservoirs of unique genetic variation for this endangered species. Protecting and restoring habitat connectivity in this region should therefore be considered a priority to maintain the evolutionary potential and long-term viability of *O. gerti*.

Conclusion

Our study provides new insights into the conservation biology of the endangered *Ophryophryne gerti* by documenting the newly confirmed population in the Di Linh Plateau. By combining distributional, morphological, ecological, and genetic data, we demonstrate that populations in the Di Linh Plateau harbor substantial genetic diversity and unique haplotypes, underscoring their importance as a conservation unit. The findings suggest an association between *O. gerti* and forested headwater streams, with leaf-litter accumulations potentially serving as important microhabitats for the larval stage. The replacement of natural riparian forest by agricultural land uses in the Di Linh Plateau appears to be associated with changes in stream structure, including increased flow intensity and reduced organic matter retention, which may potentially reduce habitat suitability for this species. Although further quantitative analyses are needed to assess population-level impacts, the findings highlight the potential vulnerability of *O. gerti* to riparian forest alteration. Conservation efforts in the region should therefore prioritize the protection and restoration of riparian evergreen forests along headwater streams to maintain the structural and ecological conditions required by stream-dependent amphibians. Future studies incorporating additional occurrence data and environmental predictors will be necessary to model habitat suitability and further refine conservation priorities for *Ophryophryne gerti* in southern Vietnam.

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Additional information

Conflict of interest

The authors have declared that no competing interests exist.

Ethical statement

No ethical statement was reported.

Use of AI

No use of AI was reported.

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Author contributions

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Data availability

All of the data that support the findings of this study are available in the main text.

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